

**CO-CULTURE OF EQUINE MESENCHYMAL STEM CELLS WITH
SYNOVIOCYTES TO MODULATE INDUCED INFLAMMATION
IN-VITRO**

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Co-Culture of Equine Mesenchymal Stem Cells with Synoviocytes to Modulate Induced
Inflammation In-Vitro
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Stem cells are the current topic of interest when it comes to the forefront of tissue engineering and new noninvasive therapies; and at the pinnacle of regenerative medicine for orthopedic tissues and immunomodulatory treatments stands Mesenchymal Stem Cells (MSC). MSCs have demonstrated multiple therapeutic uses due to their abilities to both induce tissue regeneration in multiple mesoderm derived cell lineages, as well as respond to and mediate cellular activity in their local environment.

In order to better understand the ways in which MSCs act to mediate acute inflammation, an environmental scenario similar to that of osteoarthritis was created by inducing synovitis in-vitro. Equine synoviocytes were harvested, plated, and then exposed to the inflammatory cytokines TNF-Alpha and IL-1Beta. The synoviocytes suspended in the pro-inflammatory cytokines were then treated with autogenic MSCs in order to observe the mechanisms by which they would interact with the synoviocytes in order to regulate inflammation. The media containing both the mesenchymal stem cells and the inflamed synoviocytes was harvested and the concentration of extracellular PGE-2 was analyzed using ELISA in an effort to observe any

communication between the two cell types. Synoviocyte proliferation was also observed visually by comparing colony formation and cell confluence prior to inflammation, following inflammation, and following MSC treatment. Further investigation must be done before definitive results can be described. Significant conclusions cannot be drawn based on the preliminary data that is currently available.

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NOMENCLATURE

bFGF – Basic Fibroblast Growth Factor

BSA – Bovine Serum Albumin

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl Sulfoxide

DPBS – Dulbecco's Phosphate Buffered Saline

ELISA – Enzyme-Linked Immunosorbent Assay

FBS – Fetal Bovine Serum

FDA – Fluorescein Diacetate

HBSS – Hanks Buffered Saline Solution

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IL-1B – Interleukin – 1 Beta

ITS+ - Insulin, Transferrin, Selenium Cell Culture Supplement

MSC – Mesenchymal Stem Cell

MSCi-SF – Serum Free MSC Isolation Media

PBS – Phosphate Buffered Solution

PGE2 – Prostaglandin E2

SIM – Synoviocyte Isolation Media

TCEP – tris(2-carboxyethyl)phosphine

TNF-A – Tumor Necrosis Factor - Alpha

Tris – (Hydroxymethyl)-aminomethane

CHAPTER I

INTRODUCTION

Mesenchymal Stem Cells (MSCs), also known as Marrow Stem Cells, Multipotent Stromal Cells or Mesenchymal Sticky Cells, are multi-potent stromal cells that can differentiate into multiple cell lineages including the orthopedic tissues composed of adipocytes, chondrocytes, and osteocytes.^{1,3} During embryonic development, MSCs arise during embryonic trunk development from SOX1(+) neuroepithelial cells in the mesoderm.² Morphologically, MSCs are characterized by their fibroblast-like appearance, with long slender cell processes and a small cell body.⁴ As stem cells, MSCs are characterized by their ability to undergo self-renewal while maintaining their multipotency.⁷ While the majority of MSCs are derived from red bone marrow, other sources of MSCs include muscle tissue, adipose tissue, amniotic fluid, umbilical cord blood, and a variety of other tissues.⁷ In horses, MSCs are most often harvested from the bone marrow of sternum. The primary method of isolating MSCs from a population of marrow derived cells is attributed to their novel ability to adhere to plastic.¹ Other cells can be washed from the isolate before the MSCs are collected by trypsinization.

MSC populations are also characterized by the expression of specific cell surface markers which can be identified with the use of a flow cytometer. The accuracy of these specific cell surface markers in representing a population of MSCs, and what precise characteristics are expressed by a true MSC are currently topics of debate. However, it is generally accepted that expression of the cell surface markers STRO-1, VCAM-1, CD-105, CD-90, and CD-73, and the lack of

expression of MHC-II, CD-79a, CD-45, CD-34, CD-19, CD-14, and CD-11b, is representative of a population of mesenchymal stem cells.^{1,5,7}

Since MSCs have the ability to generate multiple lines of mesoderm derived tissues, it is obvious that their primary application is in tissue regeneration and tissue engineering. MSCs have successfully demonstrated these regenerative abilities on multiple occasions in a variety of different situations; from repairing tendons and cartilaginous defects, to being used to engineer customized bones, to being inserted in absorbable matrices to repair tendons and ligaments, to treatment of osteogenesis imperfect in children.^{6,11-15} Once MSCs are injected, exogenous MSC populations are recruited to site of an injury by the presence of signaling cytokines such as SDF-1.^{10,16} By the same mechanism, endogenous MSCs located in their natural niche are mobilized to assist in natural tissue repair.^{9,10,16}

In addition to their regenerative abilities, MSCs are also known for their pleiotropic nature, being able to respond to stimuli from their local environment via both direct cell to cell contact and contact independent mechanisms.³ Because of this, MSCs possess the ability to regulate their local environment and mediate inflammation via paracrine activity, secreting cytokines and growth factors that have a variety of roles in stimulating endogenous cell populations.^{8,9} The mechanisms by which this is done include suppressing the immune system by regulating T-Cell proliferation, inhibiting fibrosis and apoptosis, and stimulating angiogenesis, cell proliferation, and cell differentiation to enhance tissue repair.^{3,8,9} The pro-inflammatory cytokines in the tumor necrosis factor family and interleukin family, such as TNF- α and IL-1 β , are commonly found in particularly abundant concentrations in the synovial lining of inflamed joints.³ One of the proposed mechanisms in which MSCs regulate acute inflammation in their microenvironment is by the secretion cytokines such as prostaglandin (PGE-2).^{3,16} Because of their ability as trophic-

mediators, MSCs could potentially be powerful tools when it comes to future treatment methods for joint inflammation and diseases such as rheumatoid arthritis and gout.¹⁷

The possible clinical applications for MSCs are vast, ranging from utilizing their regenerative capabilities in order to combat defects in orthopedic tissue, to utilizing their anti-inflammatory properties to ameliorate the symptoms of arthritis.^{3,17} This experiment will focus primarily on the mechanisms in which MSCs act to mediate inflammation and communicate with other cells in their local environment. Specifically, how MSCs respond when exposed to an environment of synoviocytes in either TNF-A or IL-1B in-vitro. A better understanding of how these cells act in-vitro will help in developing future clinical applications for MSCs in-vivo.

CHAPTER II

METHODS AND MATERIALS

Equine synoviocytes were isolated from the synovial membrane of the metacarpo(tarso)phalangeal joints of horses euthanized for reasons unrelated to this study. The synovial membrane was treated with 10mL/g of digest media consisting of 1.5mg/mL Collagenase, 0.15mg/mL of DNase, and synoviocyte isolation media (SIM), consisting of 500ml of High Glucose DMEM mixed with 12.5mL of 1M HEPES, 5mL of 200mM L-Glutamine, and 5mL of sodium pyruvate, along with 50mL of fetal bovine serum (FBS) and 5mL of Penicillin/Streptomycin (10,000 IU/mL Pen, 10,000ug/mL Strep stock) and plated for 2-3 days until confluent. The plated synoviocytes were then treated with 5mL of trypsin to remove them from the plastic flask and treated with 5mL of 10% equine serum in order to neutralize the trypsin and protect the synoviocyte cells from proteolytic cleavage. The synoviocytes were then rinsed with 10 ml of DPBS and cryopreserved in a solution consisting of 10mL of FBS, 10mL of dimethyl sulfoxide (DMSO), and 80mL of SIM until use.

Autologous mesenchymal stem cells were isolated from bone marrow collected from the sternum of each of the horses used for collecting synovium. Raw marrow was spun down and erythrocytes were lysed using 1X Tris-NH₄Cl Lyses Solution (pH of 7.2) which consisted of 7.7g of NH₄Cl and 2.06g of Tris (Hydroxymethyl)-aminomethane dissolved in 1L of deionized water. Bone marrow was treated with the lyses solution twice in proportions of 20mL lyses solution per mL of bone marrow. Cells were centrifuged and lyses solution was aspirated between treatments. Remaining lyses solution was aspirated and the cells were then suspended in 10mL of MSC

isolation media consisting of 500mL of Low-Glucose (1g/dL Glucose) DMEM mixed with 12.5mL of 1M HEPES, 5mL of 200mM L-Glutamine, 5mL of sodium pyruvate and 5mL of fungizone. The MSCs were then plated in T175 flasks and suspended in an equal volume of MSC isolation media (without fungizone) in order to expand cell populations. The MSCs were incubated at 37 degrees Celsius for 7 to 10 days until the cells grew into a 70% confluent monolayer. During that time media was replaced approximately every 48 hours. Once the cells reached confluence, media was aspirated and the flask was washed with 10mL of HBSS. Cells were then treated with 5mL of trypsin and incubated for 5 minutes to speed up separation of the cells from the flask. The cells were then treated with 5mL of HBSS to neutralize the trypsin. Lastly, the cells were aspirated from the flask, centrifuged to remove the supernatant, and cryopreserved in a solution composed of 10mL FBS, 10mL DMSO, and 80mL MSC isolation media.

A stock solution of sterile 0.1% bovine serum albumin (BSA) in 1X Phosphate Buffered Solution (PBS) was made under a laminar flow hood. The inflammatory cytokines TNF-A and IL-1B were reconstituted in the stock solution of 0.1% BSA in 1X PBS to concentration of 10ng/uL for each. Three six well Corning plates were labeled for each of the 3 conditions tested: Synoviocytes Only, MSC Synoviocyte Co-Culture, and MSCs Only. For each of the three plates, two wells were designated for each of the inflammatory conditions: TNF-A, IL-1B, and Control.

Synoviocytes were thawed, re-suspended in DPBS, and spun down at 300G for 4 minutes. Following this initial rinse the DPBS was then aspirated off and the synoviocytes were re-suspended in 20 mL of SIM. 100uL of the synoviocyte cell suspension was added to a solution composed of 60uL of propidium iodide, 740uL of HBSS, and 100ul of a mixture of 10uL of fluorescein diacetate (FDA) and 2.5mL of DPBS. The synoviocytes were then counted using a

hemocytometer and fluorescent microscopy. Initial counts were used to estimate the number of live cells recovered post thaw by multiplying the number of live cells by the volume of SIM they were suspended in and then 10,000 to account for the sample size measured by the hemocytometer. Following counting, 100,000 synoviocytes were plated in each of the spot wells of the “Synoviocytes Only” plate, and 50,000 synoviocytes were plated in each of the spot wells of the “MSC Synoviocyte Co-Culture” plate. Each spot well received a volume of cells not to exceed 2 mL and additional SIM was added so that the volume in each spot well was exactly 2 mL. The “MSCs Only” spot wells received only 2mL of SIM. Cells were incubated at 37 C for 8-12 hours following plating.

Plates were inspected at 40x and 100x magnification following incubation. Media from each spot well was then aspirated and each spot well was rinsed with 2mL of Hank’s Balanced Salt Solution (HBSS) before the addition of 2 mL of serum free MSC Isolation media (MSCi-SF), consisting of 500mL of Low-Glucose (1g/dL Glucose) DMEM (also mixed with 12.5mL of 1M HEPES, 5mL of 200mM L-Glutamine, and 5mL of sodium pyruvate) along with 5mL of ITS+ (.01X Concentration of Stock), 5uL of (1ng/mL) bFGF, 5mL Penicillin/Streptomycin (50,000 U Antibiotic) and 1mL of Amphotericin B. Each of the spot wells were then inflamed with 1 uL of their respective inflammatory cytokine reconstituted in 0.1% BSA in 1X PBS. Control wells received only 1 uL of the stock solution of 0.1% BSA in 1X PBS. The synoviocytes were incubated at 37 C for 4-6 hours while being exposed to the inflammatory mediators. Spot wells were again inspected after the 4-6 hour exposure period.

In a separate vial, autogenic MSCs were thawed, re-suspended in DPBS, and spun down at 300G for 4 minutes. The DPBS was aspirated off and the MSCs were re-suspended in 20 mL of MSCi-SF. The MSCs were then counted using the same procedure for the synoviocytes. Following

counting, 100,000 MSCs were plated in each of the spot wells for the “MSCs Only” plate, and 50,000 MSCs were plated in each of the spot wells for the “MSC Synoviocyte Co-Culture” plate. Each spot well received a volume of cells not to exceed 1 mL and additional MSCi-SF was added so that the volume added to each spot well was exactly 1mL. The “Synoviocyte Only” spot wells received 1mL of only MSCi-SF. The plates were then incubated for 24 hours at 37 C. After the 24 hour incubation period, media was harvested in 560uL aliquots in 1.5mL microcentrifuge tubes. The tubes were then frozen at -20 C and the remainder of the media was aspirated.

Protein analysis was done via ELISA using a Prostaglandin E2 Parameter Assay Kit from R&D Systems. 2 and 3, and later 5 and 10 fold dilutions of the harvested media were done prior to running each ELISA plate. Concentration of PGE2 was measured by observation of the relative absorbance at 450nm following binding of fluorescent conjugate.

CHAPTER III

RESULTS

Thus far, ELISA data for all of the horses co-cultured has been both inaccurate and inconclusive. No pattern in absorbance was expressed between the different dilution factors of each of the samples or by the stepwise dilution of the PGE-2 standard provided by the kit. Because of the inconsistency of the ELISA plates to provide From the current ELISA data, the only observation that can be made is that higher concentrations of PGE-2 were detected for each conditions of the “MSC Only” culture when compared to the “Synoviocyte Only” and “Co-Culture.” However, the actual concentration of PGE-2 in the samples cannot yet be quantified, and the actual significance, if any, cannot be determined.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2500	2500	TNF-1L	TNF-1L	C-1L	C-1L	IL-1L	IL-1L	TNF-1L	TNF-1L	C-1L	C-1L
B	1250	1250	TNF-1H	TNF-1H	C-1H	C-1H	IL-1H	IL-1H	TNF-1H	TNF-1H	C-1H	C-1H
C	625	625	TNF-2L	TNF-2L	C-2L	C-2L	IL-2L	IL-2L	TNF-2L	TNF-2L	C-2L	C-2L
D	313	313	TNF-2H	TNF-2H	C-2H	C-2H	IL-2H	IL-2H	TNF-2H	TNF-2H	C-2H	C-2H
E	156	156	IL-1L	IL-1L	TNF-1L	TNF-1L	C-1L	C-1L	IL-1L	IL-1L		
F	78	78	IL-1H	IL-1H	TNF-1H	TNF-1H	C-1H	C-1H	IL-1H	IL-1H		
G	39	39	IL-2L	IL-2L	TNF-2L	TNF-2L	C-2L	C-2L	IL-2L	IL-2L		
H	0	0	IL-2H	IL-2H	TNF-2H	TNF-2H	C-2H	C-2H	IL-2H	IL-2H	NSB	NSB

Table 1 - ELISA Plate Layout

Black (1-2:A-H): Standard Dilution

Blue (5-6:E-H, 7-8:A-H): Synoviocytes Only

Red (3-4:A-H, 5-6:A-D): Co-Culture

Green (9-10:A-H, 11-12:A-D): MSCs Only

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.198	0.236	0.172	0.27	0.218	0.226	0.226	0.213	0.541	0.445	0.606	0.511
B	0.16	0.376	0.144	0.207	0.199	0.287	0.228	0.163	0.478	0.476	0.508	0.306
C	0.195	0.297	0.167	0.252	0.23	0.292	0.156	0.15	0.5	0.216	0.567	0.273
D	0.185	0.425	0.324	0.209	0.19	0.247	0.211	0.297	0.488	0.342	0.561	0.515
E	0.183	0.196	0.162	0.164	0.165	0.254	0.231	0.153	0.588	0.455		
F	0.172	0.198	0.152	0.162	0.229	0.271	0.236	0.17	0.484	0.304		
G	0.082	0.063	0.333	0.205	0.247	0.421	0.243	0.168	0.446	0.391		
H	0.024	0.041	0.168	0.313	0.356	0.273	0.506	0.492	0.489	0.43	0.002	-0.002

Table 2 – ELISA Absorbance at 540nm (Corrected)

(Comparison of the absorbance of different dilutions exemplifies the inaccuracy of the ELISA plate)

From visual inspection of the cultures, it appears that the cells of the “Synoviocyte Only” culture are healthier compared to the “Co-Culture” MSCs and synoviocyte cells. Likewise, the cells in the “MSC Only” culture appear healthier and more confluent compared to the cells of the Co-Culture.

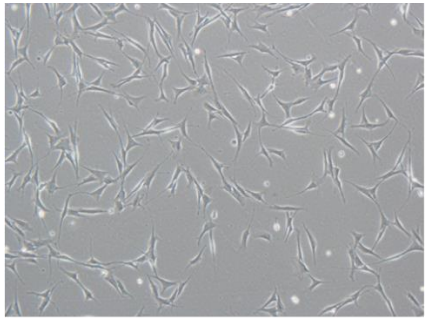


Figure 1 – “Synoviocytes Only”
Pre-Inflammation (T = 24 Hours)

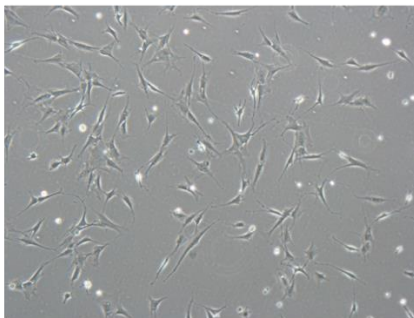
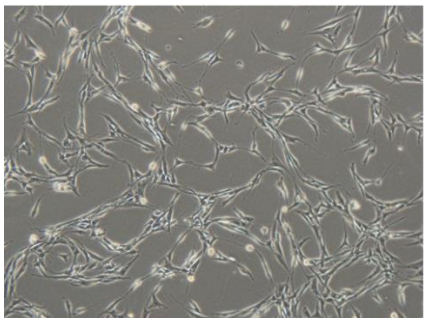


Figure 2 (Left) – “Synoviocytes Only”
Post-Inflammation (T = 48 Hours)
Control

Figure 3 (Right) – “Co-Culture”
Post-Inflammation (T = 48Hours)
Control

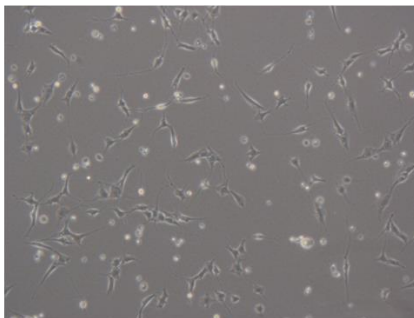
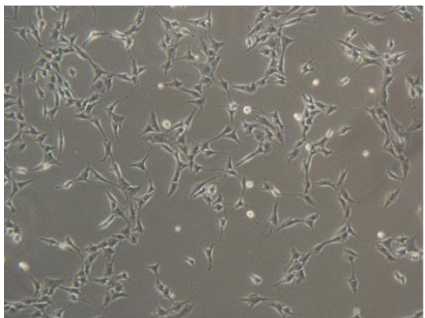


Figure 4 (Left) – “Synoviocytes Only”
Post-Inflammation (T = 48 Hours)
IL-1 β

Figure 5 (Right) – “Co-Culture”
Post-Inflammation (T = 48Hours)
IL-1 β

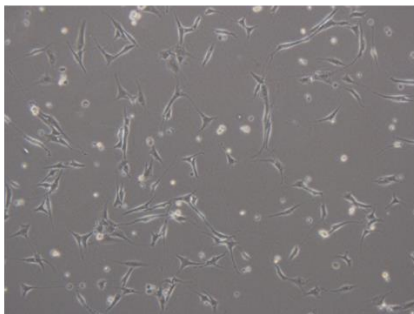
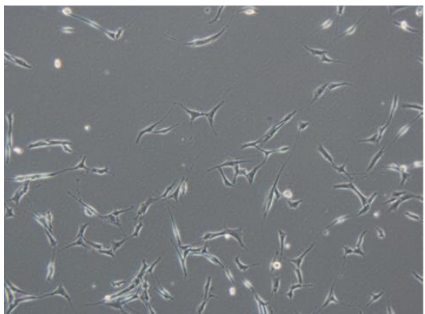


Figure 6 (Left) – “Synoviocytes Only”
Post-Inflammation (T = 48 Hours)
TNF- α

Figure 7 (Right) – “Co-Culture”
Post-Inflammation (T = 48Hours)
TNF- α

CHAPTER IV

DISCUSSION

While the reported data is inconclusive, it is important to remember that results from this experiment are still preliminary. ELISA plates for these preliminary horses will be redone using a kit from a different supplier. Hopefully, differences between the different cell cultures, as well as differences between the inflammatory conditions will then be observed. Statistical significance will be determined once accurate data is generated and compiled for the new ELISA plates.

From this experiment, it was expected that the inflamed synoviocytes co-cultured with MSCs would have higher concentrations of PGE-2, since it is suspected that MSCs secrete PGE-2 as a paracrine mechanism to communicate with cells in their local environment in order to reduce the prevalence of pro-inflammatory cytokines. Also, minimal PGE-2 would ideally be observed in the “synoviocyte only” condition which will prove that secretion of PGE-2 and inflammatory regulation is specifically attributed to MSC activity. However, from the preliminary ELISA data, it appears that the media of the “MSC Only” culture contained the highest concentrations of PGE-2. While this does follow the latter hypothesis that secretion of PGE-2 will be attributed to MSC activity in mediating the local environment, it does not explain why the amount of PGE-2 is not similarly elevated in the Co-Culture media. One possible explanation for this initial observation is that the MSCs in the “MSC Only” culture did not have any cells to communicate with in their microenvironment. For this reason, the cells secreted more PGE-2 in an effort to mediate the environment when no change in stimuli occurred following the initial secretion of PGE-2. Another possible explanation for the observed elevated concentrations of PGE-2 may be

due to the conditions established by the experimental protocol. The “MSC Only” culture had 100,000 MSCs plated while the “Co-Culture” had only 50,000 MSCs plated. Despite this, it would still be expected that there would be an intermediate elevated level of PGE-2 concentration in the “Co-Culture” when compared to the concentrations of PGE-2 in the “MSC Only” and “Synoviocyte Only” cultures. A possible continuation to this experiment would be to observe another Co-Culture in which the synoviocytes are treated with 100,000 MSCs for comparison against the “MSC Only” culture.

Similar logic can be applied to give a possible explanation for the observed visual differences between the “Co-Culture” and “Synoviocyte Only” cultures. It was expected that in this experiment the inflamed synoviocytes in the “Co-Culture” would appear healthier and more confluent following treatment with MSCs due to the MSCs mediating the effects of the pro-inflammatory cytokines in the media. However, it appears that the synoviocytes in the Co-Culture are less healthy and less confluent compared to the “Synoviocyte Only” culture. A possible explanation for the observed difference may again be due to the difference in the number of cells that were plated in the different cultures. Only 50,000 synoviocytes were plated in the “Co-Culture” while 100,000 synoviocytes were plated in the “Synoviocyte Only” culture. For this reason, the “Synoviocyte Only” culture may appear to be healthier and more confluent simply because twice as many synoviocytes were plated to begin with.

Following ELISA, this experiment will be continued by using qRT-PCR in order to observe what transcripts are present in the cell lysate. From this, the products that are being actively transcribed by the cells can be determined. This, in conjunction with the results from ELISA, will give a more definite reference as to how MSCs are acting under the applied conditions to mediate their environment.

Lastly, this experiment can be improved upon by positively identifying the isolated cell populations prior to the co-culture portion of the experiment. Flow cytometry can be used in order to determine that the isolated cells express the cell surface markers for either MSCs or synoviocyte cells. Validation that these cell populations are being used is necessary to determine that MSCs are the cells responsible for any observed differences between the different cell culture conditions. Overall, further investigation is required before conclusions can be made based on the experimental results.

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